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(54) A monoclonal antibody recognizing a cytotoxin, a hybridoma cell line expressing same and a process for the preparation of a purified cytotoxin.

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(73) Proprietor: YEDA RESEARCH AND DEVELOPMENT COMPANY LIMITED
P.O. Box 95
Rehovot 76100(IL)

(72) Inventor: Wallach, David
14, Hadar Street
Rehovot(IL)

(74) Representative: Patentanwälte Grünecker, Kinkeldey, Stockmair & Partner
Maximilianstrasse 58
W-8000 München 22(DE)

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Description

The present invention relates to a hybridoma cell line expressing a monoclonal antibody which specifically recognizes and binds a cytotoxin having a M.W. of 17,000 ± 500 D as determined by polyacrylamide SDS gel electrophoresis, a monoclonal antibody which specifically recognizes and binds said cytotoxin, a process for the preparation of a purified cytotoxin having a M.W. of 17,000 ± 500 D as determined by polyacrylamide SDS gel electrophoresis, a solid phase immunoassay, by which multiple hybridoma cultures can be screened for the production of antibodies which can bind said cytotoxin, and a process for preparing monoclonal antibody against said cytotoxin.

Background of the invention:

Proteins which exert a toxic effect on cells were found to be secreted, in response to stimulation, by mononuclear cells of various kinds. T-cells, of probably both the helper and the suppressor subsets can respond to antigens recognized by them, as well as to mitogenic lectins, by secreting such cytotoxic proteins (Granger, G.A. and Kolb, W.P., J.Immunol. 101, 111-120 (1968); Ruddle, N.H. and Waksman, B.H. J. Exp. Med. 128, 1267-1275 (1968); Eardley, D.D., Shen, F.W., Gershon, R.K. and Ruddle, N.H., J. Immunol. 124, 1199-1202 (1980)). Monocytes and macrophages produce cytotoxic proteins in response to certain bacterial toxins (reviewed by Ruff, M.R. and G.E. Gifford in Lymphotoxins, E. Pick and M. Landy editors, Academic Press, Inc. New York, 235-272, (1981)). Natural killer cells secrete cytotoxic proteins upon incubation with appropriate target cells (Wright, S.C. and Bonavia, B., J. Immunol. 129, 433-439, (1982)) while cells of certain continuous B lymphocyte lines were found to produce spontaneously cytotoxic proteins (Rosenau, W., Stites, D. and Jemtrud S., Cell. Immunol. 43, 235-244, (1979)). Proteins produced in lymphocyte cultures are usually referred to as "lymphotoxins", while the term "tumor necrosis factor" is often used for cytotoxic proteins produced in cultures of monocytes or of macrophages. Evidence has been presented that such cytotoxic proteins can selectively destroy tumor cells (Rundel, J.O. and Evans, C.H., Immunophormacol. 3, 9-18, (1981). So far, only a single protein of this type, produced spontaneously by cells of a B lymphocyte line has been characterized in some detail. It was purified to homogeneity and its molecular weight was estimated to be about 20,000 daltons (Aggarwal, B.B., Moffat, B. and Harkins, R.N., J. Biol. Chem. 259, 686-691 (1984)).

Summary of the invention:

There is provided a process for preparing a purified, essentially homogeneous CT, naturally produced by peripheral mononuclear blood calls. The purified CT has a M.W. of about 17,000 ± 500 Daltons as determined by SDS gel electrophoresis.

CT can be isolated by the use of monoclonal antibodies against such CT which can be obtained form mice injected with partially purified or crude preparations of CT. There is provided a technique for establishing lines of lymphocytes producing such anti-CT antibodies. Such lines are advantageously established by screening a plurality of hybridomas derived from splenocytes of such immunized mice. There is also provided a monoclonal antibody specific for CT. Such monoclonal antibody is produced by such hybridoma cell lines and is used for isolating CT in substantially homogeneous purified form.

The thus obtained purified cytotoxin, CT, is recognized by a specific anti-CT antibody. A therapeutically effective amount of at least said cytotoxin or a salt thereof may be used for the preparation of a medicament for selectively treating virus infected cells.

The term "salt" refers to salts of either or both the carboxyl and the amino groups of CT, and the term "derivatives"- to covalent modifications of the polypeptide side chains of the CT. The nature of the carrier for the CT, salt or derivative depends on the way it is applied for therapeutic purposes - be it in the form of a cream or lotion - for topical application or in the form of liquid, in which the CT, salt of derivative will be stabilized by adding components such as human serum albumin, for injection or for oral application.

The said CT is effectively cytotoxic to cells in the presence of metabolic blockers such as eveloheximide (CHI), Actimomycin D or Mitomycin C but in the absence of these agents cells of many kinds exhibit resistance to its cytotoxic effect. Injection by viruses can also render cells vulperable to killing by the said CT.

Compositions comprising the said CT effectively enhance, for example, the killing of VSV infected SV-80 cells while having no cytotoxic effect on uninfected SV-80 cells. Killing of virus infected cells by the said CT is potentiated by IFNs, primarily by IFN-γ, when those are applied at substantial concentrations. Thus, in applying the said CT containing compositions for therapy, pharmaceutical compositions containing also a suitable IFN are of advantage, and so are also pharmaceutical compositions containing metabolic blockers such as CHI or Actimomycin D or Mitomycin C.

The purified CT is effectively cytotoxic to tumor and to virus infected cells at concentrations as

low as 10 picograms/ml. The amounts of CT applied for therapy will be adjusted to reach such range of concentrations, or higher ones, in the target tissues.

Hybridoma cells producing the antibodies against CT were deposited with the International Culture Collection of Institute Pasteur, Paris, France, under Deposition No. 1-472, deposited on July 16, 1985, designated by us as Cell Line CT.

Description of the invention:

The invention is illustrated in the following by way of example. Fig. 1 is a scheme illustrating the technique of isolation of CT. As shown the process steps involve immunizing a suitable laboratory animal (mice, etc.) with preparations enriched with CT by chromatographic procedures, followed by monitoring serum titers of CT-neutralizing and CT-binding antibodies by the techniques set out in Fig. 2a and 2b. Hybridomas derived from splenocytes of the immunized mice were screened for the production of CT-binding antibodies by the procedures set out in Fig. 3. Hybridomas found to produce such antibodies were cloned and the monoclonal antibodies produced were applied to immunoadsorbent columns on which CT was affinity-purified from preparations of lymphokines which had been induced in PBMC by concanavalin A (Con A) and phorbol-12-O-myristate 13 acetate (TPA) and then partially purified by chromatography on controlled-pore glass. The critical step was the screening of a large number of hybridoma cultures for detecting a few producing antibodies against CT. The technique developed for that purpose (set out in Fig. 3) involves a solid phase CT-binding assay, which allows a rapid screening of hybridoma cultures for the presence of such antibodies, followed by a bioassay by which CTs bound to the solid phase can be sensitively detected, using cells sensitized to the cytotoxic effect of CT by cycloheximide.

Fig. 4. demonstrates the selectivity in the binding activity of a monoclonal antibody thus isolated, comparing CT-binding to a binding of interferon-γ by immunoadsorbents constructed from this antibody (A), as well as from two other unrelated monoclonal antibodies (B, C). It shows that of these three antibodies only one binds CT, namely, that which is directed against CT (CT-1 in Fig. 4A). It also shows that this binding of CT occurs without binding any detectable amounts of another protein in the cytotoxin preparation-IFN-γ. Under the same conditions of experiment a monoclonal antibody against the latter, shown in B, does bind effectively IFN-γ without binding CT at all.

Fig. 5 shows in C the CT purified on an immunoadsorbent constructed from the monoclonal antibody, as detected by Coomassie blue staining, following electrophoresis on an acrylamide gel in the presence of SDS. (Also shown is the pattern of proteins in the crude preparation of lymphokines from which this CT has been purified, in A, the lack of any binding of protein when applying this crude preparation on an immunoadsorbent constructed from an irrelevant antibody, (against DNP) in B and molecular weight standards, in D). Fig. 6 shows how the molecular weight of this CT is estimated by comparison to the mobility, on that acrylamide gel, of the standard proteins shown in Fig. 5D. A selective cytotoxic effect of the CT and its enhancement by IFN are demonstrated in Fig. 7 which shows the cytotoxic effect of the CT at various concentrations on VSV-infected SV-80 cells (●) and its further enhancement by treating these cells with IFN-γ (10 U/ml, 16 hr prior to infection (o) or 100 U/ml, prior to infection (Δ)) in comparison to the resistance to CT observed in uninfected cells (■) even when they are also treated with IFN-γ at 100 U/ml (□).

Description of the preferred Embodiment:

The following example is given for illustration only.

1. Induction of CT: Human peripheral blood mononuclear cells (PBMC) are isolated on "Ficoll-Hypaque" (Pharmacia, Upsala, Sweden) from the "buffy-coats" of freshly donated blood and depleted of platelets by differential centrifugation. The cells are suspended at a concentration of 10^7 cells/ml and incubated at 37°C in MEM alpha medium (Gibco, Grand Island, N.Y.). CT is induced in these cells by one of the following techniques:

A. Preparations used for the immunization of mice are induced by stimulating PBMC with phytohemagglutinin-P (PHA). Prior to that stimulation the cells are first incubated for 12 h in the presence of a crude preparation of lymphokines (0.2 µg/ml). This treatment, does not result in the production of CT but greatly increases the responsiveness of the cells to subsequent stimulation. PHA (5 µg/ml) (Difco, Detroit) is then added and the PBMC are further incubated for 24 h. The medium is then collected, centrifuged at 2500 rpm for 15 min to remove cell debris, and processed for concentrating and enriching the CT as described below.

B. Preparations of lymphokines used for purifying CT on immunoadsorbents are advantageously induced with Con-A as it was found difficult to fully eliminate traces of PHA in the purification procedure. The cells are first treated for 12 h with 0.25 µg/ml Con-A. At this concentration Con-A does not induce sig-

nificant secretion of CT but it increases the responsiveness of the cells to subsequent stimulation by a higher concentration of Con-A. Phorbol-12-O-myristate 13 acetate (TPA) is then added to a concentration of 5 ng/ml. and 3 h later Con-A us added to concentration of 10 µg/ml. The cells are incubated for 24 h and then, following replacement with fresh media containing 5 µg/ml Con-A for a further period of 24 h. The media are combined and centrifuged, methyl α -D Mannoside (Sigma, St. Louis, Mo.) is added to a concentration of 50 mM and the media are then further processed for purification on the immunoadsorbent as described below.

C. Alternatively the CT can effectively be induced in human peripheral-blood mononuclear cells, in monocytes isolated from the mononuclear cell population or in cultured cells such as U9 37 whose properties resemble those of monocytes by applying to these cells Sendai virus (200 HAU/ml) and incubating the cells for a period of about 12 hours to allow the production of CT. The cell media are then centrifuged and processed for purification of the CT as described below.

2). Quantitation of CT: CT is quantitated by determining its cytotoxic effect by a bioassay (Wallach, D., J. Immunol. 132, 2464-2469 (1984)). Samples to be tested are applied in several serial dilutions simultaneously with the application of cycloheximide (CHI 50 µg/ml) into micro-wells containing confluent cultures of the SV-80 cells. The extent of cell killing, determined by measuring the uptake of neutral-red by the cells, is quantitated 20 hours later, by using a MicroELISA Autoreader (Dynatech, Alexandria, VA).

3. Chromatographic Enrichment of CT: Crude preparations of CT are first concentrated by adsorption to controlled pore glass (CPG) (PG-350-200 Sigma St. Louis, MO) followed by desorption in 0.5 M tetramethyl ammonium chloride (TMAC) and then further concentrated by ultrafiltration with an Amicon PM-10 membrane (Amicon, Denvers, MA). CT preparations applied for immunization of mice are then further purified by one of the two following procedures:

(A) CPG-concentrated CT preparations are fractionated by electrophoresis on 7.5% acrylamide gels, under non-denaturating conditions (Walker, S.M. and Lucas, Z.J., J. Immunol. 113, 813-823, (1974), Lewis, J.E., Carmack, C.E., Yamamoto, R. and Granger, G.A., J. Immunol. Meth. 14, 163-176 (1977)). Fractions eluted from slices of the gels, which exhibit cytotoxic activity are pooled, concentrated by ultrafiltration on a PM-10® membrane and injected into mice.

(B) CPG-concentrated CT preparations are equilibrated with 1 M NACl, 30% ethylene glycol, 10 mM sodium phosphate and 0.1 mM EDTA and subjected twice, sequentially, to fractionation on Ultrogel® AcA44. Following each fractionation, fractions exhibiting cytotoxic activity are pooled and concentrated on a PM-10® membrane. The cytotoxic proteins recovered from the second run on the Ultrogel column are applied to further purification by preparative isoelectrofocusing on a 1% ampholine gradient (pH 3.5-10) constructed in sucrose solution using an LKB 8100-1® column. Fractions exhibiting maximal cytotoxic activity, peaking at about pH 6.4 are pooled, concentrated, equilibrated with PBS and then injected into mice.

Immunization with CT and Cell Fusion:

Four month old female CB6 mice are injected with samples of 10 µg of CT preparations - five injections with CT enriched by procedure A, as described above, and another two injections with CT enriched by procedure B. In the first immunization, the proteins are emulsified in complete Freund's adjuvant and injected into the foot pads of the mice (0.5 ml/mouse). The second injection, is given 3 weeks later, and the rest of the injections which are given at 1 to 2 week intervals, are all given subcutaneously using alumina gel as adjuvant (0.3 µg/0.25 ml/mouse). Immunization is then discontinued for a month and the mouse showing the highest titer of serum antibodies against CT is injected twice, intraperitoneally, at a 1 day interval, with 10 µg of a CT preparation enriched by procedure B. A day after the second immunization, the mouse is sacrificed and its splenocytes are fused with myeloma cells. The fused cells are distributed into multiple wells of microtiter plates and hybridomas are selected for in HAT-containing tissue culture medium. Hybridomas found to produce antibodies against CT are cloned in soft agar. For growing these cells in the ascitic fluid of mice they are inoculated intraperitoneally at 10^7 cells per mouse 2-4 weeks following intraperitoneal injection of 0.5 ml pristan.

Quantitation of Antibodies against CT in Mouse Sera and in Hybridoma Growth Media:

The level of antibodies against CT in sera of mice is determined by measuring their neutralizing and binding activities.

CT Neutralizing Activity: (Fig. 2a)

Samples of CT (10 U in 50 µl Dulbecco's modified Eagle's medium containing 2% FCS (DMEM-2% FCS)) are incubated for 4 h at 37° with samples of mouse sera (50 µl), serially diluted in DMEM-2% FCS. They are then further incubated for 12-16 h at 4°C and than assayed for CT activity at eight 2-fold dilutions.

CT Binding Activity: (Fig. 2b)

Samples of crude concentrated CT (30 µl, 10^4 U/ml) are incubated for 4 h at 37°C in conical-bottom micro-titer wells (Greiner) with samples of the mouse serum, serially diluted in DMEM-2% FCS. Normal mouse serum (20 µl of a 1:40 dilution in PBS) is added, followed by 60 ul of goat antiserum against mouse $F(ab)'_2$. The plates are further incubated for 30 min at 37°C and then overnight at 4°C and are then spun at 1200 g for 5 min at 4°C. The immunoprecipitates are rinsed twice with cold PBS and once with unbuffered saline, solubilized by adding 50 µl NH_4OH and assayed for CT activity at eight 2-fold dilutions.

The Solid Phase Assay for Detecting CT Binding Monoclonal Antibodies:

(applied in screening the hybridoma growth media for the presence of CT-binding antibodies, Fig. 3). PVC microtiter plates (Dynatech, Alexandria, VA) are incubated, with affinity purified goat antibody against mouse $F(ab)_2$ (80 µg/ml in PBS, 80 ul/well) then with samples of the hybridoma growth media (50 µl/well) and finally with samples of a crude concentrated CT preparation (10^4 U/ml, 50 µl/well). Each of the incubation periods is for 12-18 h (at 4°C and following each the plates are rinsed 3 times with PBS. The plates are then further rinsed once with unbuffered saline and the bound CT is dissociated by applying NH_4OH (75 mM containing 0.1% FCS 20 µl/well). A hundred ul of 0.04 M Na-Hepes pH 7.4 in DMEM-10% FCS are added and the eluted cytotoxic activity is quantitated on CHI-sensitized SV80 cells, at four, two-fold dilutions.

Purification of CT on Immunoadsorbents:

Monoclonal antibodies are purified from ascitic fluids by precipitation with ammonium sulphate (50%). Those of the IgM isotype are further purified by dialysing against water followed by solubilization precipitating IgM in PBS. 10 mg of each of the immunoglobulins are coupled to 1 g Trisacryl GF2000® (LKB) which was derivitized with aminocaproic acid and activated with N-hydroxy succinimide. Uncoupled antibody is removed by washing the resin with 50 mM Na-citrate pH 2.8 and then with 0.15 NH_4OH.

For purification of CT on the immunoadsorbent, samples of 0.5 ml of the resin are mixed for 2 h at 4° C with 3 ml of CT preparation in the presence of 0.5 M TMAC. The resins are then packed in small columns, unbound protein is washed with 0.5 M TMAC solution. The columns are then further washed with 0.5% NP-40 in 0.5M TMAC, then with a solution of 1 M Nacl, containing also 10 mM sodium phosphate buffer pH 7.4 and then with unbuffered saline and the bound CT is eluted by applying 0.2 M NH_4OH and neutralized with 1 M acetic acid within 10 min of elution. All steps of the immunoaffinity purification procedure are carried out at 4°C.

Analysis of the Purified CT by SDS Gel Electrophoresis:

Fig. 5. shows the pattern of proteins in a crude preparation of cytotoxins as analyzed on SDS-polyacylamide gel (15%). Ammonia eluted fraction from an immunoadsorbent constructed from the antibody U13-6 (against DNP) on which the crude CT has been applied (in B). CT purified from the crude preparation of the CT-1 immunoadsorbent column (in C) and molecular weight standard (phosphorylase 94K, bovine serum albumin 67K, ovalbumin 43K, carbonic anhydrase 30K, soybean trypsin inhibitor 20.1K and lysozyme 14.4K daltons (in D) as shown in Fig. 6. Fig. 5c shows that the purified CT constitutes a single polypeptide species. The molecular weight of the purified protein as estimated by comparison to the mobility on the acrylamide gel of other proteins with known molecular weights, is about 17.5Kd in Fig. 6.

Claims

1. A hybridoma cell line expressing a monoclonal antibody which specifically recognizes and binds a cytotoxin having M.W. of 17,000 ± 500 D as determined by polyacrylamide SDS gel electrophoresis said hybridoma being formed by fusion of murine myeloma cells with spleen cells from a mouse previously immunized with a pure or impure preparation of a human cytotoxin obtained from stimulated monocytes or monocyte-like cells, which is specifically recognized and bound by the reference monoclonal antibody produced by the hybridoma cell line CNCM I-472 deposited with the Institute Pasteur.

2. The hybridoma cell line of claim 1 being the cell line CNCM I-472.

3. A monoclonal antibody which specifically recognizes and binds a cytotoxin having M.W. of

17,000 ± 500 D as determined by polyacrylamide SDS gel electrophoresis.

4. A monoclonal antibody according to claim 3 produced by a hybridoma cell line according to claim 1.

5. A monoclonal antibody of the type IgM said antibody being produced by the hybridoma cell line according to claim 2.

6. A process for the preparation of a purified cytotoxin having M.W. of 17,000 ± 500 D as determined by polyacrylamide SDS gel electrophoresis, which comprises:
(a) Providing a preparation containing the cytotoxin;
(b) adsorbing the cytotoxin from said preparation onto controlled pore glass beads;
(c) desorbing the cytotoxin in a state of enhanced purity from said controlled pore glass beads;
(d) contacting the desorbed cytotoxin with an immunoadsorbent, said immunoadsorbent comprising a monoclonal antibody according to any of claims 3 to 5, and
(e) eluting the cytotoxin from the immunoadsorbent.

7. The process of claim 6 wherein the cytotoxin is desorbed in step c by means of a desorption buffer including 0.5 M tetramethyl ammonium chloride.

8. The process of claim 6 or 7 wherein the cytotoxin is eluted from the immunoadsorbent by means of about 0.2 M NH_4OH.

9. The process of any of claims 6 to 8 where the preparation of step (a) is made from stimulated peripheral blood mononuclear cells.

10. The process of any of claims 6 to 8 where the cells are stimulated with phytohemagglutinin after incubation with lymphokines.

11. The process of any of claims 6 to 9 where the cells are stimulated by concanavalin-A after incubation with a concentration of concanavalin-A that does not itself induce significant secretion of cytotoxin.

12. The process of any of claims 6 to 9 in which the cells are stimulated with Sendai virus.

13. A process for the preparation of a purified cytotoxin having M.W. of 17,000 ± 500 D as determined by polyacrylamide SDS gel electrophoresis which comprises contacting a preparation containing a cytotoxin having M.W. of 17,000 ± 500 D as determined by polyacrylamide SDS gel electrophoresis with an immunoadsorbent comprising a monoclonal antibody as defined in claim 5 and eluting the cytotoxin from said immunoadsorbent.

14. A solid phase immunoassay by which multiple hybridoma cultures can be screened for the production of antibodies which can bind cytotoxin having M.W. of 17,000 ± 500 D as determined by polyacrylamide SDS gel electrophoresis which comprises:
(a) Coating protein-binding support means with affinity purified antibody against mouse immunoglobulins;
(b) incubating the tested hybridoma growth media in the coated support means followed by washing;
(c) incubating samples of cytotoxin in the support means followed by washing;
(d) dissociating the cytotoxin which has bound to the support means and determining its amount in a bioassay.

15. The immunoassay of claim 14 in which said bioassay comprises providing cells sensitized to the cytotoxic effect of the cytotoxin by means of a metabolic blocker, adding the cytotoxin and measuring the extent of cell death.

16. The immunoassay of claim 15 in which the metabolic blocker is cycloheximide.

17. The immunoassay of claim 15 in which the metabolic blocker is actinomycin D.

18. The immunoassay of claim 15 in which the metabolic blocker is mitomycin C.

19. A process for preparing monoclonal antibodies against a cytotoxin having M.W. of 17,000 ± 500 D as determined by polyacrylamide SDS gel electrophoresis, which comprises immunizing mice with either pure or impure preparations of such protein, detecting hybridomas which produce such antibodies by means of the immunoassay of any of claims 15 to 18, cultivating such hybridomas and obtaining the desired antibodies.

20. The process of claim 19 in which the cells used in the bioassay of step d. of claim 14 are cycloheximide-sensitized SV80 cells.

21. A process for isolation of a cytotoxin having

M.W. of 17,000 ± 500 D as determined by polyacrylamide SDS gel electrophoresis which comprises:

(a) Preparing a monoclonal antibody according to any of claims 3 to 5 against the cytotoxin following immunization with impure preparations of the cytotoxin;

(b) constructing an immunoadsorbent from these antibodies and using the same for purifying the cytotoxin from crude preparations thereof.

22. Use of a therapeutically effective amount of at least a cytotoxin having M.W. of 17,000 ± 500 D as determined by polyacrylamide SDS gel electrophoresis or salt thereof in essentially homogenous form, for the preparation of a medicament for treatment of virus infected cells.

Patentansprüche

1. Hybridomzellinie, die einen monoklonalen Antikörper exprimiert, der ein Zytotoxin mit einem Molekulargewicht von 17000 +/- 500 D, bestimmt mittels SDS-Polyacrylamidgelelektrophorese, spezifisch erkennt und bindet, wobei das Hybridom durch Fusion einer Mäusemyelomzelle mit Milzzellen einer Maus gebildet ist, die zuvor mit einer reinen oder unreinen Präparation eines aus stimulierten Monozyten oder monozytenähnlichen Zellen erhaltenen menschlichen Zytotoxins immunisiert worden ist, wobei das Zytotoxin von einem monoklonalen Referenzantikörper, der von der beim Pasteurinstitut hinterlegten Hybridomzelllinie CNCM I-472 erzeugt wird, spezifisch erkannt und gebunden wird.

2. Hybridomzellinie nach Anspruch 1, wobei die Hybridomzellinie die Zellinie CNCM I-472 ist.

3. Monoklonaler Antikörper, der ein Zytotoxin mit einem Molekulargewicht von 17000 +/- 500 D, bestimmt mittels SDS-Polyacrylamidgelelektrophorese, spezifisch erkennt und bindet.

4. Monoklonaler Antikörper nach Anspruch 3, erzeugt von einer Hybridomazellinie nach Anspruch 1.

5. Monoklonaler Antikörper von Typ IgM, wobei der Antikörper von einer Hybridomzellinie gemäß Anspruch 2 erzeugt wird.

6. Verfahren zum Herstellen eines gereinigten Zytotoxins mit einem Molekulargewicht von 17000 +/- 500 D, bestimmt mittels SDS Polyacrylamidgelelektrophorese, umfassend:

a) Bereitstellen einer das Zytotoxin enthaltenden Präparation;

b) Adsorbieren des Zytotoxins aus der Präparation an Glasperlen bestimmter Porengröße (controlled pore glass beads);

c) Desorbieren des Zytotoxins in einem Zustand erhöhter Reinheit von den Glasperlen mit bestimmter Porengröße;

d) Inkontaktbringen des desorbierten Zytotoxin mit einem Immunadsorptionsmittel, wobei das Immunadsorptionsmittel einen monoklonalen Antikörper gemäß einem der Ansprüche 3 bis 5 umfaßt, und

e) Eluieren des Zytotoxins von dem Immunadsorptionsmittel.

7. Verfahren nach Anspruch 6, wobei das Zytotoxin in Schritt c) mittels eines Desorptionspuffers, der 0,5 M Tetramethylammoniumchlorid enthält, desorbiert wird.

8. Verfahren nach Anspruch 6 oder 7, wobei das Zytotoxin von dem Immunadsorptionsmittel mittels ungefähr 0,2 M NH_4OH eluiert wird.

9. Verfahren nach einem der Ansprüche 6 bis 8, wobei die Präparation aus Schritt a) aus stimulierten peripheren mononukleären Blutzellen gemacht wird.

10. Verfahren nach einem der Ansprüche 6 bis 8, wobei die Zellen nach Inkubation mit Lymphokinen mit Phytohämagglutinin stimuliert werden.

11. Verfahren nach einem der Ansprüche 6 bis 9, wobei die Zellen nach Inkubation mit einer Concanavalin-A-Konzentration, die für sich selbst keine signifikante Sekretion des Zytotoxins induziert, mit Concanavalin-A stimuliert werden.

12. Verfahren nach einem der Ansprüche 6 bis 9, in dem die Zellen mit Sendai-Virus stimuliert werden.

13. Verfahren für die Herstellung eines gereinigten Zytotoxins mit einem Molekulargewicht von 17000 +/- 500 D, bestimmt mittels SDS-Polyacrylamidgelelektrophorese, das das Inkontaktbringen einer Präparation, enthaltend ein Zytotoxin mit einem Molekulargewicht von 17000 +/- 500 D, bestimmt mittels SDS-Polyacrylamidgelelektrophorese, mit einem Immunadsorptionsmittel, umfassend einen monoklonalen Antikörper, wie in Anspruch 5 definiert, und Eluieren des Zytotoxins von dem Immunad-

sorptionsmittel umfaßt.

14. Festphasenimmuntest, mittels dessen eine Vielzahl von Hybridomkulturen auf die Erzeugung von Antikörpern untersucht werden kann, die ein Zytotoxin mit einem Molekulargewicht von 17000 +/- 500 D, bestimmt mittels SDS-Polyacrylamidgelelektro- phorese, sezernieren, umfassend:
a) Beschichten einer proteinbindenden Trägervorrichtung mit affinitätsgereinigtem Antikörper gegen Mausimmunglobulin;
b) Inkubieren der untersuchten Hybridomwachstumsmedien in der beschichteten Trägervorrichtung, gefolgt von Waschen;
c) Inkubieren von Proben des Zytotoxins in der Trägervorrichtung, gefolgt von Waschen;
d) Dissoziieren des Zytotoxins, das an die Trägervorrichtung gebunden hat, und Bestimmen seiner Menge in einem biologischen Test.

15. Immuntest nach Anspruch 14, in dem der biologische Test das Bereitstellen von Zellen, die für die zytotoxische Wirkung des Zytotoxins mittels eines metabolischen Blockers sensibilisiert sind, das Zufügen des Zytotoxins und das Messen des Ausmaßes des Zelltods umfaßt.

16. Immuntest nach Anspruch 15, in dem der metabolische Blocker Cycloheximid ist.

17. Immuntest nach Anspruch 15, in dem metabolische Blocker Actinomycin D ist.

18. Immuntest nach Anspruch 15, in dem der metabolische Blocker Mitomyzin C ist.

19. Verfahren zum Herstellen monoklonaler Antikörper gegen ein Zytotoxin mit einem Molekulargewicht von 17000 +/- 500 D, bestimmt mittels SDS-Polyacryamidgelelektrophorese, umfassend das Immunisieren von Mäusen mit entweder reinen oder unreinen Präparationen eines solchen Proteins, das Nachweisen von Hybridomen, die solche Antikörper erzeugen, mittels des Immuntests gemäß einem der Ansprüche 15 bis 18, das Kultivieren solcher Hybridome und Erhalten der erwünschten Antikörper.

20. Verfahren nach Anspruch 19, in dem die in dem biologischen Test von Schritt d) gemäß Anspruch 14 verwendeten Zellen cycloheximid-sensibilisierte SV 80-Zellen sind.

21. Verfahren zum Isolieren eines Zytotoxins mit einem Molekulargewicht von 17000 +/- 500 D, bestimmt mittels SDS-Polyacrylamidgelelektrophorese, umfassend:
a) das Herstellen eines monoklonalen Antikörpers gemäß einem der Ansprüche 3 bis 5 gegen das Zytotoxin nach Immunisieren mit unreinen Präparationen des Zytotoxins;
b) das Konstruieren eines Immunadsorptionsmittels aus diesen Antikörpern und Verwenden desselben zum Reinigen des Zytotoxins aus rohen Zytotoxinpräparationen.

22. Verwendung einer therapeutisch wirksamen Menge mindestens eines Zytotoxins mit einem Molekulargewicht von 17000 +/- 500 D, bestimmt mittels SDS-Polyacryamidgelelektrophorese, oder eines Salzes davon im wesentlichen homogener Form für die Herstellung eines Medikamentes zur Behandlung virusinfizierter Zellen.

Revendications

1. Lignée de cellules hybridomes exprimant un anticorps monoclonal, qui reconnaît spécifiquement et lie une cytotoxine ayant un poids moléculaire de 17 000 ± 500 daltons, comme déterminé par électrophorèse sur gel de polyacrylamide et de dodécyl sulfate de sodium (SDS), cet hybridome étant formé par la fusion de cellules de myélomes de souris avec des cellules de rates en provenance d'une souris précédemment immunisée avec une préparation pure ou impure d'une cytotoxine humaine obtenue de monocytes ou de cellules analogues à des monocytes stimulés, laquelle cytotoxine est spécifiquement reconnue et liée par l'anticorps monoclonal de référence produit par la lignée de cellules hybridomes CNCM I-472 déposée à l'Institut Pasteur.

2. Lignée de cellules hybridomes selon la revendication 1, qui est la lignée de cellules CNCM I-472.

3. Anticorps monoclonal, qui reconnaît spécifiquement et lie une cytotoxine ayant un poids moléculaire de 17 000 ± 500 daltons, comme déterminé par électrophorèse sur gel de polyacrylamide et de SDS.

4. Anticorps monoclonal selon la revendication 3, produit par une lignée de cellules hybridomes selon la revendication 1.

5. Anticorps monoclonal du type IgM, cet anticorps étant produit par la lignée de cellules

hybridomes selon la revendication 2.

6. Procédé pour préparer une cytotoxine purifiée ayant un poids moléculaire de 17 000 ± 500 daltons, comme déterminé par électrophorèse sur gel de polyacrylamide et de SDS, qui comporte les stades suivants :
(a) procurer une préparation contenant la cytotoxine;
(b) adsorber la cytotoxine en provenance de cette préparation sur des billes de verre à pores contrôlés;
(c) désorber la cytotoxine dans un état de plus grande pureté de ces billes de verre à pores contrôlés;
(d) mettre en contact la cytotoxine désorbée avec un immuno-adsorbant, cet immuno-adsorbant contenant un anticorps monoclonal selon l'une des revendications 3 à 5; et
(e) éluer la cytotoxine de l'immuno-adsorbant.

7. Procédé selon la revendication 6, dans lequel la cytotoxine est désorbée au stade (c) au moyen d'un tampon de désorption contenant du chlorure de tétraméthyl ammonium 0,5 M.

8. Procédé selon la revendication 6 ou la revendication 7, dans lequel la cytotoxine est éluée de l'immuno-adsorbant au moyen de NH_4OH environ 0,2 M.

9. Procédé selon l'une des revendications 6 à 8, dans lequel la préparation du stade (a) est réalisée à partir de cellules mononucléaires du sang périphérique stimulées.

10. Procédé selon l'une des revendications 6 à 8, dans lequel les cellules sont stimulées par la phytohémagglutinine après incubation avec des lymphokines.

11. Procédé selon l'une des revendications 6 à 9, dans lequel les cellules sont stimulées par la concanavalin-A après incubation avec une concentration de concanavalin-A qui n'induit pas par elle-même une secrétion significative de cytotoxine

12. Procédé selon l'une des revendications 6 à 9, dans lequel les cellules sont stimulées par le virus de Sendaï.

13. Procédé pour la préparation d'une cytotoxine purifiée ayant un poids moléculaire de 17 000 ± 500 daltons, comme déterminé par électrophorèse sur gel de polyacrylamide et de SDS, qui consiste à mettre en contact une préparation contenant une cytotoxine ayant un poids moléculaire de 17 000 ± 500 daltons, comme déterminé par électrophorèse sur gel de polyacrylamide et de SDS, avec un immuno-adsorbant contenant un anticorps monoclonal, comme défini dans la revendication 5, et à éluer la cytotoxine de cet immuno-adsorbant.

14. Essai immunologique en phase solide, par lequel on peut cribler des cultures d'hybridomes multiples pour la production d'anticorps qui peuvent lier une cytotoxine ayant un poids moléculaire de 17 000 ± 500 daltons, comme déterminé par électrophorèse sur gel de polyacrylamide et de SDS, qui consiste à :
(a) revêtir un support de liaison des protéines avec un anticorps purifié par affinité dirigé contre les immunoglobulines de souris;
(b) faire incuber le milieu de croissance d'hybridomes testés dans le support revêtu, cette opération étant suivie par un lavage;
(c) faire incuber des échantillons de cytotoxine dans le support, cette opération étant suivie par un lavage;
(d) dissocier la cytotoxine qui s'est liée au support et déterminer sa quantité dans un essai biologique.

15. Essai immunologique selon la revendication 14, dans lequel l'essai biologique consiste à procurer des cellules sensibilisées à l'effet cytotoxique de la cytotoxine au moyen d'un bloqueur métabolique, à ajouter la cytotoxine et à mesurer l'importance des destructions de cellules.

16. Essai immunologique selon la revendication 15, dans lequel le bloqueur métabolique est le cycloheximide.

17. Essai immunologique selon la revendication 15, dans lequel le bloqueur métabolique est l'actinomycine D.

18. Essai immunologique selon la revendication 15, dans lequel le bloqueur métabolique est la mitomycine D.

19. Procédé pour préparer des anticorps monoclonaux dirigés contre une cytotoxine ayant un poids moléculaire de 17 000 ± 500 daltons, comme déterminé par électrophorèse sur gel de polyacrylamide et de SDS, qui consiste à immuniser des souris avec des préparations, soit pures, soit impures, d'une telle protéine, à détecter des hybridomes qui produisent ces anticorps au moyen de l'essai immunologique

selon l'une des revendications 15 à 18, cultiver ces hybridomes et obtenir les anticorps désirés.

20. Procédé selon la revendication 19, dans lequel les cellules utilisées dans l'essai biologique du stade (d) de la revendication 14 sont des cellules de SV80 sensibilisées par le cycloheximide.

21. Procédé pour isoler une cytotoxine ayant un poids moléculaire de 17 000 ± 500 D, comme déterminé par électrophorèse sur gel de polyacrylamide et de SDS, qui consiste à :
(a) préparer un anticorps monoclonal selon l'une des revendications 3 à 5, dirigé contre la cytotoxine, cette opération étant suivie par une immunisation avec des préparations impures de la cytotoxine; et
(b) construire un immuno-adsorbant à partir de ces anticorps et l'utiliser pour purifier la cytotoxine à partir de préparations brutes de celle-ci.

22. Utilisation d'une quantité thérapeutiquement efficace d'au moins une cytotoxine ayant un poids moléculaire de 17 000 ± 500 daltons, comme déterminé par électrophorèse sur gel de polyacrylamide et de SDS, ou l'un de ses sels sous une forme essentiellement homogène pour la préparation d'un médicament pour le traitement de cellules infectées par un virus.

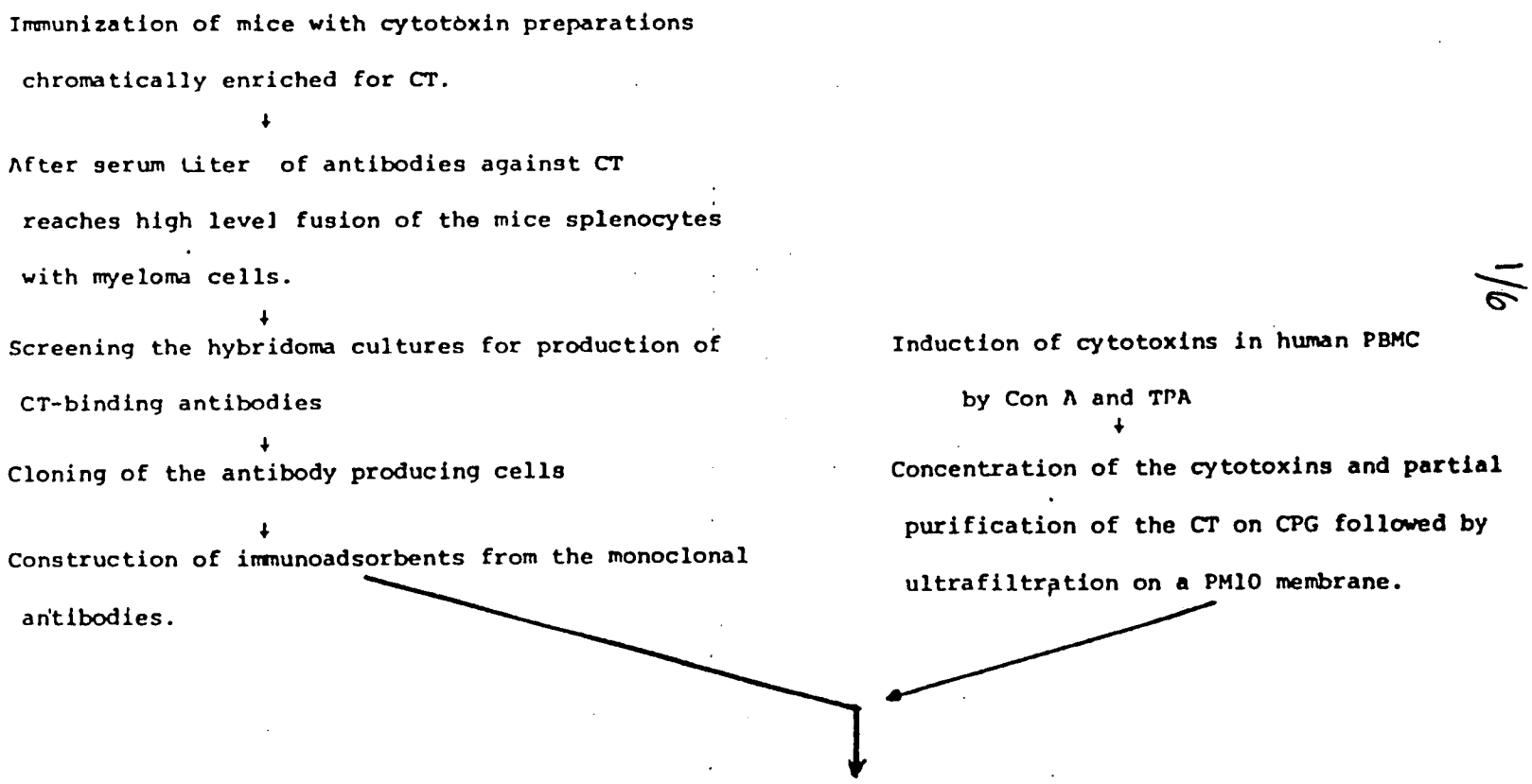

Fig. 1

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Alternative Approaches for Detection of Antibodies against CT in Sera of Immunized Animals

a Neutralization assay:	Incubation of CT preparation with antiserum.	→ Determination of CT activity (in the presence of antiserum)		
b Binding assay:	Incubation of CT preparation with antiserum	→ Precipitation of serum immunoglobulin+bound CT with goat antiserum against mouse immuno-globulins.	→ Washing of immuno precipitate followed by its solubilization at 0.075M ammonia	→ Determination of T activity in solubilized immunoprecipitate

Fig. 2

The Solid Phase Assay for Detecting, CT Binding, Monoclonal Antibodies

Adsorbtion of hybridoma produced immunoglobulin to PVC microwells which had been precoated with affinity purified antibodies against mouse immunoglobulins. → Incubation of CT preparations in microwells. → Rinsing of microwells followed by dissociation of bound antigens at 0.075M ammonia → Determination of CT activity in the proteins eluted from microwells

Fig. 3

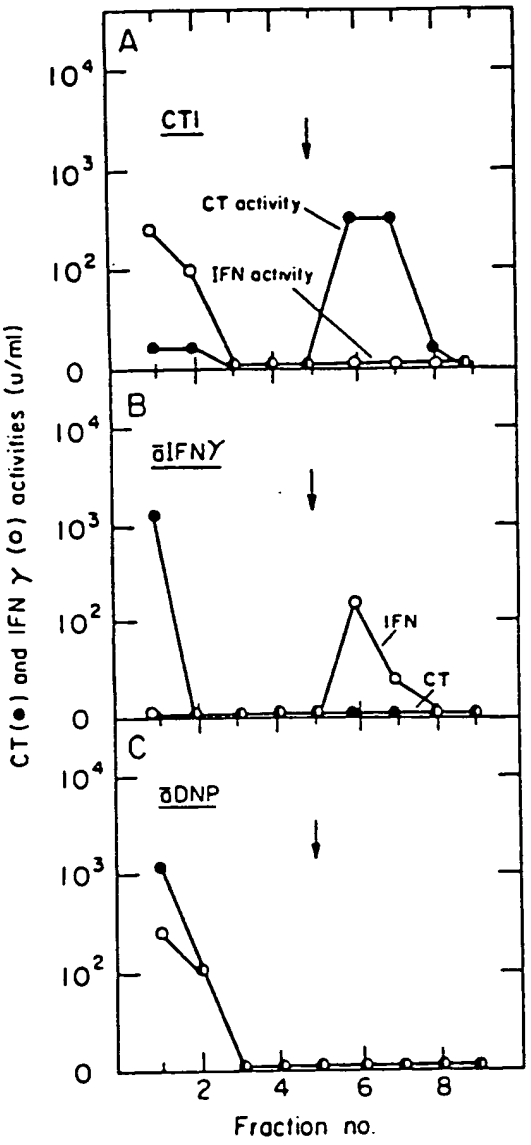
A
CTI
CT activity
IFN activity
B
αIFNγ
IFN
CT
C
αDNP
10^4
10^3
10^2
0
2
4
6
8
Fraction no.
CT (●) and IFN γ (○) activities (u/ml)

FIG 4

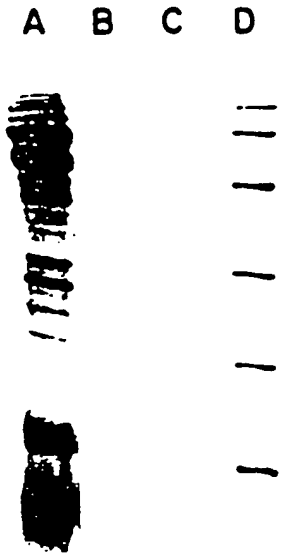
A
B
C
D

FIG 5

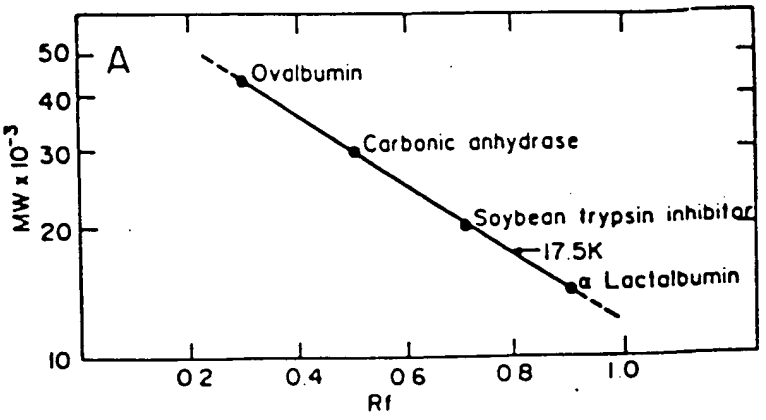
A
Ovalbumin
Carbonic anhydrase
Soybean trypsin inhibitor
17.5K
α Lactalbumin
MW x 10⁻³
50
40
30
20
10
0.2
0.4
0.6
0.8
1.0
Rf

FIG 6

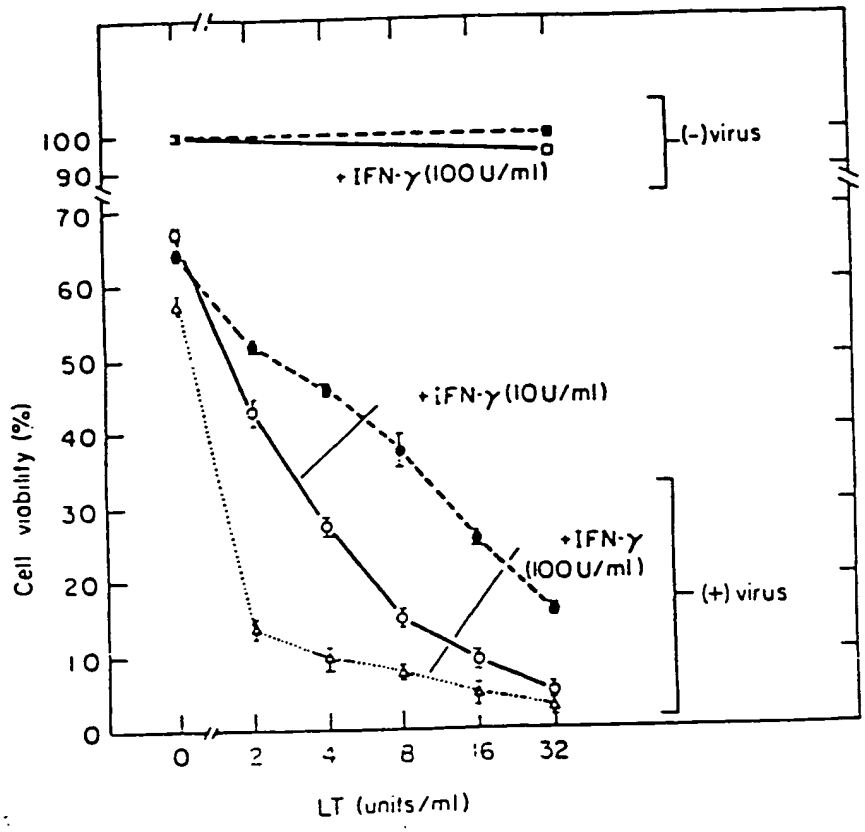
100
90
70
60
50
40
30
20
10
0
Cell viability (%)
(-)virus
+IFN-γ(100U/ml)
+IFN-γ(10U/ml)
+IFN-γ
(100U/ml)
(+) virus
0
2
4
8
16
32
LT (units/ml)

FIG. 7.

Encl. 2

Claims 3 and 5 as amended after opposition

3. A monoclonal antibody which specifically recognizes and binds a human cytotoxin having M.W. of 17,000 ± 500 D as determined by polyacrylamide SDS gel electrophoresis, which cytotoxin is recognized and bound by the reference monoclonal antibody produced by the hybridoma cell line CNCM I-472 deposited with the Institut Pasteur.

5. A monoclonal antibody, said antibody being produced by the hybridoma cell line according to claim 2.